(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 November 2002 (07.11.2002)

PCT

(10) International Publication Number
WO 02/087591 A2

(51) International Patent Classification[7]: A61K 31/585, A61P 43/00

(21) International Application Number: PCT/EP02/04211

(22) International Filing Date: 16 April 2002 (16.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
MI2001A000821 17 April 2001 (17.04.2001) IT

(71) Applicant *(for all designated States except US)*: **GIENNE PHARMA S.P.A.** [IT/IT]; Via Lorenteggio, 270/A, I-20152 Milano (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PINZANI, Massimo** [IT/IT]; Via Stefano Ucci, 11, I-50018 Scandicci (IT). **VANASIA, Massimo** [IT/IT]; Via Lorenteggio, 270/A, I-20152 Milano (IT).

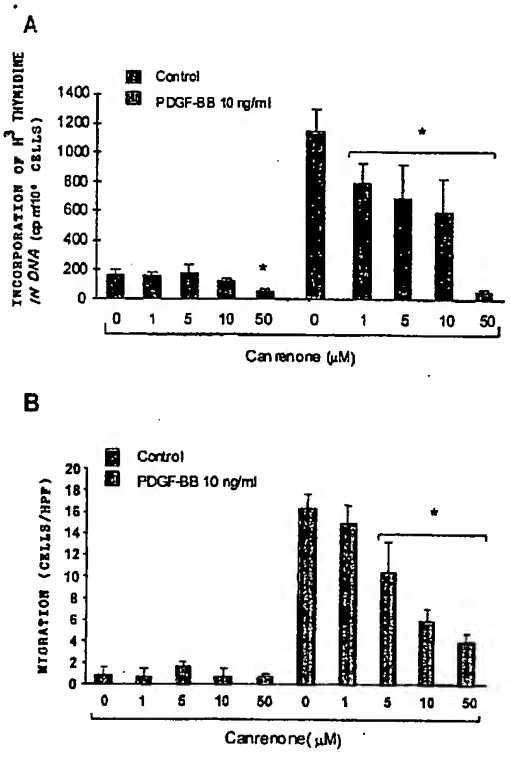
(74) Agent: **MODIANO, Guido**; Modiano & Associati, Via Meravigli, 16, I-20123 Milano (IT).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent

[Continued on next page]

(54) Title: USE OF DERIVATIVES OF SPIRONOLACTONE FOR THE INHIBITION OF THE PRO-FIBROGENIC ACTION OF HEPATIC STELLATE CELLS AND OF VASCULAR SMOOTH MUSCLE CELLS



(57) Abstract: The invention relates to the use of a spironolactone compound to produce a drug for inhibiting the pro-fibrogenic effect of hepatic stellate cells and vascular smooth muscle cells, particularly in relation to fibrogenesis induced by pro-fibrogenic cytokines. Canrenone, potassium canrenoate, spironolactone and eplerenone are particularly preferred compounds for use in the invention.

WO 02/087591 A2

(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

USE OF DERIVATIVES OF SPIRONOLACTONE FOR THE INHIBITION OF THE PRO-FIBROGENIC ACTION OF HEPATIC STELLATE CELLS AND OF VASCULAR SMOOTH MUSCLE CELLS

Technical Field

The present invention relates to inhibition of fibrogenesis progression through inhibition of the pro-fibrogenic action exerted by hepatic stellate cells and by vascular smooth muscle cells.

Specifically, the present invention relates to prevention and inhibition of cytokine-mediated fibrogenesis in hepatic stellate cells and vascular smooth muscle cells. Particularly, the present invention relates to inhibition of fibrogenesis mediated by the platelet-derived growth factor (PDGF) and/or by the transforming growth factor-β1 (TGF-β1).

Background Art

At present, fibrogenesis is regarded as a dynamic process strictly dependent on the extent and duration of parenchymal damage due to various etiologic factors. Based on this concept, some fibrogenic disorders are no longer considered degenerative, but are seen as the result of repeated tissue damage and chronic inflammation events. The process of liver fibrosis development and the processes that cause progressive tissue sclerosis in other organs and systems consist of three phases: acute inflammation, synthesis of extracellular matrix (ECM) and its remodeling, regeneration of the parenchymal cells that are typical of the tissue. According to this sequence, a single event of tissue lesion generally results in complete and effective tissue repair.

On the contrary, persistence of the initial cause of tissue damage causes chronic activation of tissue repair mechanisms. This favors the phases of the repair process that are characterized by the deposition of fibrillar matrix, with progression toward tissue fibrosis rather than toward tissue repair.

With particular reference to liver fibrosis, in qualitative terms the extracellular matrix has similar components both in a fibrotic liver and in a

normal liver. However, hepatic tissue affected by active fibrogenesis exhibits a considerable increase in ECM components, which appear altered in their relative proportions and distribution. In particular, as the fibrogenic process progresses, a considerable change in the composition of ECM along sinusoids and hepatocellular laminas is observed. There is an increased synthesis of type I and III collagens, the main components of the new structure, known as fibrillar matrix, which replaces the existing ECM that is typical of the normal liver and is characterized by a prevalence of type IV collagen and laminin. These qualitative and quantitative changes at the ECM level not only have mechanical and physical implications but also contribute to the establishment of a new biochemical environment.

The cellular origin of the components of connective tissue in cirrhotic liver has been discussed for a long time. Although different liver cell types are able to synthesize ECM components, it is currently believed that hepatic stellate cells (HSC) are the cell type that has a main role in the subsequent deposition of fibrillar ECM.

Following chronic liver damage, HSC undergo a process of activation characterized by phenotype transformation from a quiescent form to an actively proliferating one, which is morphologically similar to myofibroblast and is therefore termed "myofibroblast-like". This occurs gradually and comprises an intermediate stage defined as "transitional cell". This transformation entails morphological changes and the activation of many functional components, including increased ECM synthesis, increased expression of growth factors and cytokines and of receptors thereof, the appearance of well-developed intracellular contractile structures, and a complex alteration of the enzymatic pathways involved in the degradation and remodeling of newly synthesized ECM. In aggregate, these changes lead to a cellular phenotype characterized by remarkable pro-fibrogenic and contractile capability.

The so-called "perpetuation" of HSC activation basically consists of a

definitive transformation into the "myofibroblast-like" phenotype. The characteristics of this particular phase of HSC activation, confirmed extensively by morphological studies of cirrhotic human and rat liver, comprise a high proliferative and motile capacity, increased cell contractility and increased ECM synthesis together with a reduced ability to degrade and remodel the matrix, and increased release of cytokines and growth factors, including chemotactic agents for leukocytes (chemokines). Areas of hepatic damage attract a wide variety of inflammatory cells, which have a key role in the activation and regulation of stellate cells, stimulating their proliferation and migration as well as ECM synthesis by producing soluble factors, cytokines and growth factors. These factors, individually or as part of a complicated network of interactions, produce various effects on the biology of activated HSC.

It is important to stress that these aspects of HSC biology are basically common to other ECM producing cells present in other organs and systems, which are similarly responsible for the fibrogenic progression of diseases characterized by chronic damage and chronic inflammation. The most significant example in this regard is provided by smooth muscle cells of the subintimal layer of arteries, which are responsible for the forming of the typical vascular lesion of atherosclerosis.

Several studies performed over the last decade have highlighted the importance of various cytokines in the progression of the fibrogenic process, and particularly of two polypeptide growth factors: platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β). These factors, with different properties, are responsible for the pro-fibrogenic activation of HSC and of many other cell types responsible for disorders that have a sclerogenic evolution, such as arterial smooth muscle cells.

PDGF has biological effects on HSC, promoting their proliferation and migration, whereas TGF-β has a direct pro-fibrogenic action, increasing ECM synthesis and deposition and decreasing ECM degradation and

remodeling.

Disclosure of the invention

The aim of the present invention is to provide the use of a drug for preventing and inhibiting the progression of fibrogenesis in HSC and vascular smooth muscle cells, particularly fibrogenesis mediated by the above cytokines, by administering a drug that is already used for other clinical indications and whose side effects, within the dosage range commonly used clinically, are well established.

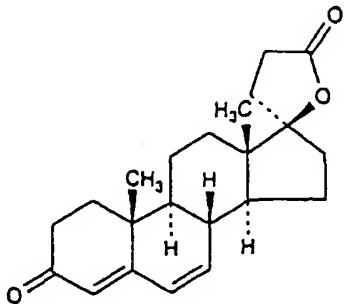
Thus a main object of the present invention is to provide the use of a drug in preventing or treating diseases such as hepatic fibrosis, vascular fibrosis, etc.

This aim and these and other objects which will become better apparent from the following description, are achieved according to the present invention by using a drug that contains a spironolactone derivative.

As used herein, the term "spironolactone" designates a molecule that comprises a lactone ring coupled with a steroid structure in spiro configuration.

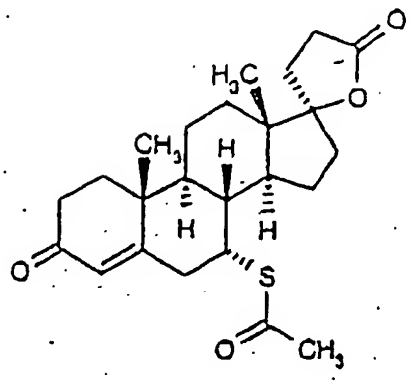
Canrenone and its prodrugs and pharmaceutically acceptable salts, such as potassium canrenoate and spironolactone itself, as well as eplerenone, are particularly preferred spironolactone compounds for use according to the present invention.

Canrenone has the following structural formula:



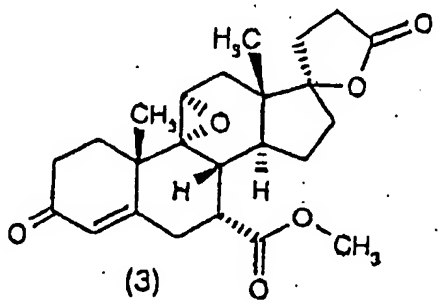
and is known as a diuretic and aldosterone antagonist.

Spironolactone has the following structural formula:



and is known as a diuretic and aldosterone antagonist.

Eplerenone, or epoxymexrenone, has the following structural formulas:



(3)

and is known as an antihypertensive and aldosterone antagonist.

The inventors have found experimentally that spironolactone compounds exert an activity of prevention and inhibition of the pro-fibrogenic role of HSC and vascular smooth muscle cells.

In particular, it has been found that spironolactone compounds exert an activity of inhibition of the pro-fibrogenic biological effects of the above mentioned cytokines.

Specifically, it has been found that spironolactone compounds, and particularly canrenone, potassium canrenoate, spironolactone itself and eplerenone, inhibit the biological effects of PDGF and TGF-β. It is important to stress that, as detailed hereinafter, these inhibitory effects are

exerted by the drug class being considered independently of their action as aldosterone receptor antagonists, and therefore with a mechanism that is absolutely original and has never been described before.

As used herein, the expressions "prevention" or "inhibition of progression" of fibrosis are employed interchangeably to include: (1) prevention or prophylactic treatment in patients with high risk of fibrosis affecting the liver, kidney and vascular system or with symptoms that suggest the possible onset of fibrosis, and (2) treatment of patients in whom fibrosis of these tissues has reached advanced progression and/or leads to evident and quantifiable clinical manifestations.

An important aspect of the present invention is that the treatment may employ low doses of spironolactone compound, and more specifically may use doses lower that the average ones commonly employed in the treatment of arterial hypertension or ascitic decompensation in patients with hepatic cirrhosis. In this manner, therapeutic effects are achievable at dosages at which side effects are minimal and electrolyte balance or water retention in the patient are substantially unaffected.

Brief description of the drawings

The invention is described in greater detail with reference to the following figures:

Figure 1 illustrates the effect of increasing doses of canrenone on DNA synthesis in basal conditions and after stimulation with PDGF-BB, measured by incorporation of [^{3}H]-thymidine into the DNA.

Figure 1B shows the effect of increasing doses of canrenone on PDGF-BB induced cell migration (chemotaxis).

Figure 2A illustrates the level of phosphorylation of the β subunit of the PDGF receptor in basal conditions and after stimulation with PDGF-BB.

Figure 2B illustrates the level of phosphorylation of PLC-γ after stimulation with PDGF.

Figure 3A illustrates the activation of ERK caused by stimulation with

PCTF.

Figure 3B shows the expression of mRNA with c-fos in basal conditions and after adding PDGF.

Figure 4 illustrates the changes in intracellular Ca^{2+} concentration after stimulation with PDGF.

Figure 5 shows the variation in intracellular pH in control conditions and in the presence of ethyl isopropyl amiloride (EIPA), a selective Na+/H+ exchanger inhibitor, or in the presence of canrenone.

Figure 6 illustrates the effect of PDGF on Na+/H+ exchanger activity and the inhibitory effect of canrenone on the activity of this exchanger induced by PDGF (panel A) in human HSC. The figure also shows that canrenone has no effect on the changes in intracellular calcium concentration induced by PDGF (panel D). Figure 6 also shows other experimental conditions aimed at demonstrating that this effect depends on an inhibition of PI 3-K activity induced by canrenone.

Figure 7, panel A, in fact shows that canrenone induces inhibition of PDGF-induced PI 3-K activity in human HSC.

Figure 8 shows that ethyl isopropyl amiloride (EIPA), an inhibitor of the Na+/H+ exchanger, inhibits proliferation and migration of human HSC, similarly to canrenone.

Figure 9 shows that incubation with canrenone (lanes 3-6) inhibits PDGF-induced focal adhesion kinase (FAK) phosphorylation in human HSC.

Figure 10 demonstrates that canrenone inhibits cell contraction coupled with intracellular calcium release induced by thrombin in human HSC.

Figure 11 shows that canrenone is capable of inhibiting de novo synthesis of type I procollagen and of other extracellular matrix components induced by TGF-β in human HSC.

Figure 12 shows that canrenone is capable of inhibiting PDGF-induced DNA synthesis and migration in smooth muscle cells isolated from the iliac

artery.

Figure 13 presents the general characteristics of the direct antifibrogenic mechanism proposed for canrenone in the cellular models employed.

The effects of spironolactone compounds on biological variations produced by cytokines were evaluated in vitro. In particular, the effects of canrenone, the active metabolite of spironolactone, on the various biological actions of PDGF on human HSC, particularly mitogenesis and chemotaxis, were evaluated.

In addition, the effects of canrenone on ECM synthesis in basal conditions and after stimulation with TGF-β were also evaluated.

The effect of canrenone on the contraction of human HSC in response to a potent vasoconstrictor such as thrombin was also evaluated.

It should be stressed in particular that the hormone aldosterone was not present or used in combination in any of the described experimental conditions, and that therefore the pharmacological effects of the canrenone compound have to be ascribed to a mechanism that is independent of the known action of canrenone as an aldosterone receptor antagonist.

Way of carrying out the invention

MATERIALS AND METHODS EMPLOYED

Materials

A monoclonal antiphosphotyrosine antibody supplied by UBI (Lake Placid, NY, USA) was used for Western blotting. Polyclonal antibodies against extracellular signal regulated kinase (ERK)-1, phospholipase Cγ1 (PLCγ) and FAK were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Myelin basic protein was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Protein A-sepharose was supplied by Pharmacia (Uppsala, Sweden). [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol) and methyl-[^{3}H]thymidine were purchased from New England Nuclear (Milan, Italy). Human recombinant PDGF-BB was supplied by Peprotech (Rock Hill, NJ, USA). Fura-2-AM was supplied by Calbiochem

Corp. (San Diego, CA, USA). Canrenone was supplied in micronized form by GN Pharma (Milan, Italy). A stock solution of 10^{-2} M in DMSO of this drug was prepared in sterile form and the subsequent experimental dilutions were made in sterile water. Human thrombin was supplied by Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents used were of analytical grade.

Isolation and culture of human HSC

Human HSC were isolated from surgical sections (10-20 g) of normal human liver unsuitable for transplantation as previously reported in the literature. Briefly, after combined digestion of the surgical parts with collagenase and pronase, the HSC were separated from other nonparenchymal liver cells by ultracentrifugation over gradients of stractan (Cellsep™ isotonic solution, Larex Inc., St. Paul, MN, USA). The cells were cultured in plastic flasks (Falcon, Becton Dickinson, Lincoln, New Jersey, USA) in Iscove's modified Dulbecco's medium supplemented with 0.6 U/ml insulin, glutamine (2 mM), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), antibacterial-antimycotic solution (all provided by GIBCO BRL Laboratories, Grand Island, NY, USA) and 20% fetal bovine serum (v/v) (Imperial Laboratories, Andover, UK). The cells were cultured in an atmosphere constituted by humidified air with 95% O_2 and 5% CO_2, with a constant temperature of 37 °C. The freshly isolated cells cultured in subsequent subcultures were characterized as described. The described experiments were performed by using HSC between the third and fifth serial passages, with a 1:3 distribution ratio. The experiments were conducting by using three different cell lines.

Isolation and culture of smooth muscle cells from human femoral artery

The portion of muscular tunic was removed, placed in Hanks' Balanced Salt Solution (HBSS) and maintained at 37°C. After two or three washings in HBSS, the muscular tunic portion was placed on a sterile plastic disk and residues of adventitial coat and calcifications were eliminated accurately.

The tissue was then minced and washed again with HBSS, and the resulting fragments were cultured on plastic in Iscove's complete medium containing 20% fetal bovine serum (FBS) in an amount barely sufficient to cover the surface of the flask. This medium was replaced with fresh medium the next day, carefully avoiding removal of artery fragments that adhere to the plastic, and then approximately every five days. Smooth muscle cells migrate from the tissue fragments to the plastic and slowly begin to proliferate in the surrounding area. Approximately one or two weeks after isolation, the artery fragments were removed and the adhered cells were kept in culture. In this manner, the cells proliferate, with a tendency to gather and form islets. Accordingly, trypsinization must be performed before the cells reach 100% confluence. Characterization of the resulting cell cultures was performed by detecting immunohistochemical positivity for specific cell markers such as α-smooth muscle actin (α-SMA) and desmin.

DNA synthesis determination

DNA synthesis was evaluated by means of the method of [methyl-^{3}H]-thymidine incorporation in the cell material precipitated with trichloroacetic acid. Briefly, the cells were cultured on plates with 24 wells, with a density of $2x10^4$ cells/well, with complete culture medium containing 20% fetal bovine serum (FBS). Once confluence (approximately $1x10^5$ cells/well) was reached, the cells were rendered quiescent by incubation in a serum-free/insulin-free (SFIF) culture medium for 48 hours. The cells were then incubated with or without antagonists, with the specified doses and methods, for 20 hours. The cells were then incubated for 4 hours with 1.0 μCi/ml of [methyl-^{3}H]-thymidine (6.7 Ci/mM). At the end of the pulsing period, the incorporated [methyl-^{3}H]-thymidine was measured according to the methods routinely used in our laboratory. The number of cells was evaluated in three different wells for each disk and the final result of the experiment was expressed as cpm/10^5 cells.

Analysis of cell migration

The experiments were performed by using Boyden chambers equipped with 8-μm porosity polycarbonate filters. The filters were pretreated with human type I collagen (20 μg/ml) for 30 min at 37°C) and then inserted between the upper chamber and the lower chamber. HSC cultured until confluence occurred were incubated in SFIF medium for 48 hours and then treated for 10 min with increasing concentrations of NO donors. The cells were then resuspended by bland trypsinization (0.05% trypsin/EDTA) and a fraction of the cell suspension (210 μl), which corresponds to approximately $4x10^4$ cells, was inserted in the top chamber and incubated at 37°C for 6 hours. The lower chamber was filled with SFIF medium (control) or PDGF-BB (10 ng/ml) in the presence or absence of the same concentration of NO donors used in the preincubation period. After fixing with 96% methanol and staining with Harris' hematoxylin, the cells migrated to the lower side of the filter were quantified as the number of cells in 10 high-power fields (HPF). All the experiments were performed in triplicate. Analysis of each triplicate was repeated twice, in different sessions and with different preparations of HSC. Possible cytotoxic effects where tested by means of the trypan blue exclusion test. This test constantly indicated a cell viability of more than 90%.

ERK activity assay

ERK activity was evaluated by measuring myelin basic protein kinase activity in cell lysates immunoprecipitated with anti-ERK antibody The cell monolayer was lysed in a RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 0.05% (v/v) aprotinin). Insoluble proteins were eliminated with high-speed centrifugation at 4 °C. Protein concentration in the supernatant was measured in triplicate by using a commercially available kit (Bio Rad; Hercules, CA, USA). Fractions of the total cell lysates (50 μg) were immunoprecipitated with an anti-ERK antibody and protein A sepharose. ERK activity was measured according to

methods routinely used in our laboratory.

Digital imaging analysis of intracellular calcium concentration in individual HSC

The cells were cultured until 60-70% confluence was reached in a complete medium on circular glass slides (25 mm diameter, 0.2 mm thickness) for 72 hours and were then incubated for 48 hours in SFIF medium. The cells were then loaded with 10 μM Fura-2-AM (Calbiochem Corp., San Diego, CA, USA) and 15% Pluronic F-127 for 30 min at 22°C. $[Ca^{2+}]_i$ was measured in a HEPES-$NaHCO_3$ buffer containing 140 mM NaCl, 3 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4. The images were recorded every 3 seconds and the calibration curve was obtained for each cell preparation. PDGF-BB (10 ng/ml) was added directly in the perfusion chamber directly after recording the $[Ca^{2+}]_i$ basal value. In parallel experiments, the cells were preincubated with NO donors before adding the PDGF-BB.

In the experiments aimed at evaluating the effects of canrenone on thrombin-induced variations of $[Ca^{2+}]_i$ /cell surface (cell contraction measurement), this agonist (0.3 U/ml) was added directly in the perfusion chamber after measuring the basal value of $[Ca^{2+}]_i$. To evaluate cell surface variations, spatial calibration was performed on a suitable grid in the same optical conditions as the rest of the experiment.

Northern Blot analysis

RNA was extracted according to Chomczynski and Sacchi's method. 10 μg of total RNA were fractionated by 1% agarose-formaldehyde gel electrophoresis and transferred on a nylon membrane. The procedure for DNA radiolabeling and membrane hybridization was performed according to methods routinely used in our laboratory.

Immunoprecipitation and immunoblotting

Cells were lysed with a buffer containing 50 mM Hepes, 150 mM NaCl,

1% Triton X-100, 5 mM $MgCl_2$, 1 mM EGTA, 2.0 μg/ml leupeptin, 2 mM Na_3VO_4, 10 μg/ml pepstatin A, and 2 mM phenyl methyl sulfonyl fluoride (PMSF). The lysates were then centrifuged at 14,000 rpm for 10 minutes. Immunoprecipitation, SDS-PAGE and immunoblotting procedures were performed according to methods routinely used in our laboratory. Detection of bands corresponding to the antibodies used was performed by chemiluminescence by using the ECL kit (Amersham Life Sciences, Little Chalfont, UK).

Measurement of Na^+/H^+ exchanger or antiporter activity

The activity of the Na^+/H^+ exchanger or antiporter was determined in HSC loaded with the pH-sensitive fluorescent marker bis-carboxyethylcarboxyfluorescein (BCECF). The cells were cultured on circular glass slides (25 mm diameter, 0.2 mm thickness) in a complete medium until 70% confluence was achieved and were then incubated in SFIF for 48 hours; the cells were then loaded with 2 μM bis-carboxyethylcarboxyfluorescein acetoxymethyl ester (BCECF AM) for 30 min at room temperature and then washed. Fluorescence in individual cells was studied by using an imaging analysis system. The intensity of the fluorescence of the marker obtained at 490 nm of excitation wavelength (520 nm emission) was divided by the fluorescence intensity obtained at 405 nm (520 nm emission). This fluorescence intensity ratio is correlated, by virtue of a suitable calibration curve, with the pH of the cytosol. Cytosolic pH measurements were made every 3 seconds and the behavior of cytosolic pH over time was followed for at least 20 minutes. HSC were acidified by adding nigericin (5 μM) in nominally Na^+-K^+-free N-methylglucamine buffer. The decrease in the calculated ratio indicates a decrease in cytosolic pH. Once the value of this ratio value had stabilized, administration of NaCl (30 mM) caused rapid cytosolic alkalinization, indicating restored Na+/H+ antiporter activity. Alkalinization kinetics is in fact an indicator of activity of this antiporter. Ethyl isopropyl amiloride (EIPA), a specific inhibitor of this

antiporter, was used as control.

PI 3-K activity measurement

Preparation of cell lysates and dosage of PI 3-K activity were performed according to procedures standardized in our laboratories. Briefly, the proteins were immunoprecipitated by using antiphosphotyrosine antibodies. After washing, the immunoprecipitate was resuspended in 50 µl of a solution containing 20 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, and 0.5 mmol/l EGTA. 0.5 µl of phosphatidylinositol (20 mg/ml) were added for 10 min, and the sample was incubated at 25 °C for 10 minutes. One microliter of MgCl2 (1 mmol/l) and 10 µCi of [G-32P] adenosine triphosphate were added simultaneously, and incubation was continued for 10 more minutes. The reaction was stopped by adding 150 µl of chloroform/methanol/37% HCl (10:20:0.2). Samples were extracted with chloroform and dried. Radioactive lipids were separated by thin-layer chromatography by using chloroform/methanol/30% ammonium hydroxide/water (46:41:5:8). After drying, the plate was autoradiographed. The radioactive spots were then scraped and counted in a β counter.

Metabolic labeling and immunoprecipitation of components of the extracellular matrix

To perform these experiments, human HSC were labeled metabolically with 25 µCi/ml of Trans ^{35}S-label™ (70% ^{35}S-methionine, 15% ^{35}S-cysteine) in the presence or absence of the various agonists. The method used is described by Rombouts et al. (J. Hepatology 2001, in press).

Statistical analysis of data

The results of the indicated experiments are expressed as mean ± SD. Statistical analysis was performed by analysis of variance (ANOVA) and, when the F value was significant, by Duncan's test.

EXPERIMENTS

In a first group of experiments, the effects of increasing doses of canrenone on PDGF-induced cell proliferation and migration were

evaluated. In a second group of experiments, the effects of preincubation with canrenone on ERK activity were evaluated.

In a third group of experiments, the effect of preincubation with canrenone on the activity of PI3K, a kinase for lipids and proteins activated through different pathways, was analyzed.

EFFECTS OF CANRENONE ON BIOLOGICAL EFFECTS AND ON INTRACELLULAR SIGNALING INDUCED BY PDGF IN HUMAN STELLATE CELLS

RESULTS

Effects of canrenone on biological effects and on intracellular signaling induced by PDGF in human stellate cells.

The effect of increasing doses of canrenone on DNA synthesis in basal conditions and after stimulation with PDGF-BB, measured by [^{3}H]-thymidine incorporation into DNA, was firstly evaluated. As shown in Figure 1A, preincubation (5 minutes) with this drug causes a dose-dependent inhibition of PDGF-induced mitogenesis. This effect is statistically significant starting at a 1-μM dose. In basal conditions, canrenone induces a significant reduction of DNA synthesis only at maximal doses (50 μM). Likewise, canrenone reduces dose-dependently PDGF-BB-induced chemotaxis, measured in a modified Boyden chamber system; in this case, the inhibitory effect can be observed starting from a dose of 5 μM (Figure 1B).

The effect of canrenone on intracellular signaling events directly correlated to the interaction of PDGF with its receptor was then evaluated. Figure 2A shows the level of phosphorylation of the β subunit of the PDGF receptor in basal conditions and after stimulation with PDGF-BB. As clearly shown, preincubation with canrenone (10 and 50 μM) has no effect on phosphorylation induced by PDGF.

Similar findings were obtained when evaluating the level of phosphorylation of PLC-γ, a signaling molecule that is physically associated

with the PDGF receptor. As shown by Figure 2B, canrenone fails to alter the phosphorylation of PLC-γ produced by stimulation with PDGF at any of the tested concentrations (10, 25 and 50 μM).

The effects of canrenone on PDGF-BB-induced ERK activity were then evaluated (Figure 3A). As clearly shown, stimulation of human HSC maintained in SFIF medium with PDGF causes activation of ERK after 10 minutes of incubation. Preincubation with increasing doses of canrenone (from 5 to 50 μM) has no effect on this activation. Similar results were obtained when analyzing the action of canrenone on expression of mRNA for c-fos, one of the genes activated early during the PDGF cascade. As shown in Figure 3B, expression of mRNA for c-fos, which was not detectable in basal conditions (lane 1), becomes evident 30 minutes after adding the PDGF (lane 2) and is not altered by preincubation of the cells with canrenone.

Variations in intracellular concentration of Ca^{2+} following stimulation with PDGF were then evaluated. As shown in Figure 4, in individual Fura 2-loaded human HSC stimulation with PDGF (control) induces a synchronous peak increase of Ca^{2+} over an average basal $[Ca^{2+}]_i$ level of 230 nM, followed by a long-lasting plateau, in accordance with data published previously by our group. Preincubation for 10 minutes with canrenone (10 and 25 μM) is not associated with any significant variation in the morphology of the plot, both regarding the peak increase and the plateau that follows it. However, a significant decrease in these components can be observed as a consequence of preincubation with high doses of canrenone (50 μM).

Figure 5 illustrates the change in intracellular pH (expressed as delta variation/min) in control conditions and in the presence of EIPA (10 μM, positive control) or canrenone (10 and 50 μM). It is evident that incubation with the Na+/H+ exchanger or antiporter inhibitor ethyl isopropyl amiloride (EIPA) blocks antiporter activity in basal conditions (i.e., in the absence of

stimulation with PDGF). This activity is not altered significantly by incubation with canrenone doses in the range 1-25 μM; however, analogously to what has been observed for $[Ca^{2+}]_i$ variations, an inhibitory effect is noticed at the dose of 50 μM. However, after stimulation with PDGF, doses of canrenone equal to 10 μM are able to inhibit the antiporter activity induced by PDGF (Figure 6A), analogously to what has been observed with EIPA (Figure 6B). Both experimental conditions do not alter the profile of PDGF-induced $[Ca^{2+}]_i$ (Figures 6D and 6E). These results suggest that canrenone has an inhibitory effect on Na+/H+ exchanger or antiporter activity only in conditions providing stimulation with PDGF, suggesting that in this case the action of the drug is performed by means of a molecular signaling event linking the PDGF receptor and the Na+/H+ exchanger or antiporter.

Phosphatidylinositol 3-kinase (PI 3-K) is a kinase for lipids and proteins that can be activated through different pathways, including tyrosine kinase and heterotrimeric G proteins. Since PDGF action in HSC is partially dependent on the activation of PI 3-K, and since the activity of this signaling pathway seems to have considerable implications on the activity of the Na+/H+ exchanger or antiporter, we evaluated the effects of canrenone on the activity of this kinase in this cell type. As shown by Figure 7A, stimulation with PDGF (10 ng/ml) causes an increase in PI 3-K activity and preincubation of the cells with canrenone (25 and 50 μM) reduces this activity dose-dependently. In addition, as shown in Figure 7B, a similar inhibitory effect is produced by preincubation with EIPA. The results shown in Figure 6C, which relate to preincubation with the PI 3-K inhibitor LY294002, indicate that PI 3-K inhibition leads to reduced activity of the exchanger or antiporter, analogously to what has been observed with canrenone. It is therefore conceivable that canrenone affects PDGF-induced Na+/H+ exchanger or antiporter activity through PI 3-K inhibition.

As a consequence of the results obtained in the experiments described

above, we evaluated whether canrenone and EIPA had similar effects on proliferation, differentiation and cell migration mechanisms. In particular, we evaluated the action of these substances on DNA synthesis and chemotaxis induced by PDGF in human HSC. As shown by Figure 8A, canrenone (10 and 50 μM) and EIPA (5 and 10 μM) do not alter basal DNA synthesis, yet both cause dose-dependent inhibition of PDGF-induced DNA synthesis.

Similar results were obtained in experiments aimed at evaluating chemotaxis; as clearly shown in Figure 8B, preincubation of the cells with EIPA (5 and 10 μM) and canrenone (10 μM) causes a marked reduction in PDGF-induced cell migration.

Finally, we evaluated whether canrenone and EIPA modulated phosphorylation of FAK, a key protein of the focal adhesion complex. As shown in Figure 9, adhesion of HSC to plastic is associated with FAK phosphorylation; cell stimulation with PDGF-BB (for 10 minutes) causes an increase in the phosphorylation of this protein. Incubation of cells with canrenone (10 and 50 μM) and EIPA (10 μM) reduces FAK phosphorylation both in basal conditions and after stimulation with PDGF.

Effects of canrenone as vasodilator drug in human HSC

We then evaluated the effect of canrenone on the changes in $[Ca^{2+}]_i$ associated with variations in cell area in response to stimulation with thrombin, a potent vasoconstrictor. As shown in Figure 10A, stimulation with 0.3 U/ml thrombin produces a rapid increase in $[Ca^{2+}]_i$ associated with a rapid reduction in cell area, which corresponds to a reversible cell contraction coupled with variations in $[Ca^{2+}]_i$. As shown by Figure 10B, preincubation with 25 μM canrenone causes a marked reduction in these variations, suggesting an anticontractile action of this drug. Moreover, this effect is greater for higher concentrations of canrenone (50 μM), as shown in Figure 10C.

Effects of canrenone on de novo synthesis of components of the

extracellular matrix in basal conditions and after stimulation with TGF-β.

As shown in Figure 11A, incubation with 10 μM canrenone causes a significant inhibition of de novo synthesis of procollagen type I induced by TGF-β both in the cell layer and, more importantly, in the culture supernatant. A similar effect can be observed for de novo synthesis of fibronectin induced by TGF-β (Figure 11), while canrenone does not seem to significantly affect the de novo synthesis of procollagen type IV (Figure 11C).

Effects of canrenone on cell proliferation and migration induced by PDGF in smooth muscle cells isolated from human arteries an maintained in culture

In order to extend the findings obtained in human HSC, we performed a series of preliminary experiments in mesenchymal cells involved in the deposition of extracellular matrix and in the progressive sclerosis of other organs and systems. Owing to their involvement in atherosclerosis, we first of all considered the effects of canrenone in smooth muscle cells isolated from human arteries. As shown in Figures 12A and 12B, the effects of canrenone on PDGF-induced cell proliferation and migration in this cell type were similar to those observed in HSC.

CONCLUSIONS

The results of these experiments indicate that canrenone, known as a diuretic and as an aldosterone receptor antagonist, and used extensively in diuretic treatment during cirrhosis with ascites, is capable of inducing a dose dependent inhibition of the main biological effects of PDGF in human HSC, which are considered as one of the main effectors of the abnormal accumulation of fibrillar extracellular matrix as a consequence of chronic liver damage. In view of the role potentially played by this growth factor in damage repair and in the fibrogenic process at the level of the liver, these findings suggest that canrenone, as well as the other antialdosterone drugs of

which canrenone is the final metabolite, may act as an antifibrogenic drug regardless of antagonism with respect to aldosterone.

In a first set of experiments, the effects of increasing doses of canrenone on PDGF-induced cell proliferation and migration were evaluated. Although these biological actions of PDGF are not directly involved in the excessive deposition of fibrillar ECM, they are the basis for the progressive increase in HSC in the liver during active fibrogenesis and during the occupation, by this cell type, of areas of tissue in which the damage is highest and therefore the repair effort is also greatest. The results of this first set of experiments suggest that, at least in the dose range 1-10 μM, canrenone has an inhibitory effect on the action of PDGF, without affecting significantly basal proliferation and migration of HSC. This suggests a specific effect on the biological effects induced by PDGF.

The results of the first group of experiments in order to evaluate the effects of increasing doses of canrenone on PDGF-induced cell proliferation and migration show that, at least in the dose range between 1 and 10 μM, canrenone has an inhibitory effect on the action of PDGF without significantly affecting basal migration and proliferation of hepatic stellate cells (HSC). This suggests a specific effect on the biological effects induced by PDGF.

In order to understand at which level canrenone can interfere in the complex cascade of intracellular signal transduction events induced by stimulation with PDGF, we then investigated the effects of this drug on the main signaling pathways activated by this growth factor. Accordingly, we first studied the effects of canrenone on autophosphorylation of PDGF receptor type β and on phosphorylation of a signaling pathway that is physically associated with this receptor, such as PLC-γ, following stimulation with PDGF.

The results of experiments aimed at evaluating the effects of canrenone on $[Ca^{2+}]_i$ variations induced by PDGF stimulation are in agreement with the

finding of no effect of canrenone on PLC-γ phosphorylation. In particular, canrenone, at the doses of 10 and 25 μM, did not induce the variations in the peak or the plateau that normally follow stimulation with PDGF.

Together, the findings of this first group of experiments suggest that the effects of canrenone are not to be ascribed to interference with immediate post-receptor events, i.e., events that directly follow PDGF receptor phosphorylation.

Experiments regarding the effects of preincubation with canrenone on ERK activity show that activation of this pathway, located downstream of Ras, is indispensable for the progression of the signal originated by stimulation with PDGF at the nuclear level and ultimately for inducing the mitogenic effect of this growth factor. However, the results of this group of experiments have indicated that the inhibitory effect of canrenone on the action of PDGF is not exerted through inhibition of this fundamental pathway, as also confirmed by the absence of effect on mRNA expression for the c-fos protooncogene, which is a typical nuclear target for this signaling pathway.

The lack of effect of canrenone on a fundamental pathway of transduction of the mitogenic effect of PDGF such as Ras/ERK therefore led to the hypothesis of a possible action of this drug on signaling events, activated as a consequence of stimulation with PDGF and capable of modifying the cytosolic microenvironment, such as for example the induction of variations in intracellular pH. In view of this hypothesis, we evaluated the effects of canrenone on Na^+/H^+ exchanger or antiporter activity. The Na^+/H^+ exchanger or antiporter regulates intracellular levels of the hydrogen ion and, together with other membrane antiporters (HCO_3/Cl^-, Na^+/K^+-ATPase), helps to regulate intracellular pH both in basal conditions and after stimulation with growth factors, such as PDGF, as recently confirmed also in rat HSC. This set of experiments provides evidence that canrenone, although unable to inhibit the activity of the exchanger in basal conditions, is capable of

reducing exchanger or antiporter activity induced by PDGF. It is therefore conceivable that the effect of canrenone on the biological actions of PDGF is, at least partially, a consequence of an alteration of this important intracellular homeostatic system.

Therefore, we analyzed the effect of preincubation with canrenone on the activity of PI 3-K, a lipid and protein kinase activated through several different pathways, comprising tyrosine kinase and heterotrimeric G proteins, whose activation is partly responsible for the action of PDGF in HSC. It should also be noted that the activity of this signaling pathway seems to have considerable implications on the activity of the Na^+/H^+ exchanger or antiporter. Differently from the findings related to ERK, canrenone has been demonstrated to be effective in inhibiting dose-dependently the activation of this signaling pathway.

The experiments related to the effect of preincubation with canrenone on the activity of PE3K, a lipid and protein kinase activated through several different pathways, comprising tyrosine kinase and heterotrimeric G proteins, whose activation is partly responsible for the action of PDGF in HSC, showed that differently from the findings related to ERK, canrenone is effective in inhibiting dose-dependently the activation of this signaling pathway.

In this regard, when compared with the action of hitherto known inhibitors of PI 3-K activation (i.e. wortmannin), the effect of canrenone has the particularity that it does not induce a parallel, albeit partial, inhibition of ERK. The inhibitory action of canrenone on this signaling pathway can be in itself sufficient to explain interference with the biological effects of PDGF in HSC.

As shown schematically in Figure 13, the data discussed so far lead, as a whole, to the conclusion that the inhibitory effect of canrenone on PDGF actions in human HSC is most of all a consequence of an inhibition of PI 3-K and Na^+/H^+ exchanger or antiporter activity. These various actions of

canrenone may be linked, since studies conducted on different cell types have demonstrated that PDGF-induced activation of the Na^+/H^+ exchanger or antiporter is dependent on PI 3-K activation. In particular, PI 3-K activation reportedly leads to augmented recycling of the antiporter toward the plasma membrane without increasing significantly the expression of the corresponding protein. In this manner, the availability of the antiporter increases for a period of time that can be estimated in hours after stimulation with growth factors capable of activating PI 3-K. Further experimental evidence of the possible close link between PI 3-K activity and Na^+/H^+ exchanger or antiporter activity is the absolutely original finding of an inhibitory activity performed by EIPA, which is considered as a specific Na^+/H^+ exchanger or antiporter inhibitor, on the activity of PI 3-K.

Recently published studies have shown that Na^+/H^+ antiporter activity, which seems to be coordinated with the activation of Rho, a signaling molecule of the Ras superfamily involved in cytoskeletal reorganization induced by stimulation with growth factors, is important in regulating cell adhesion and in reorganizing focal adhesion complexes. In agreement with this possibility, preincubation of HSC with canrenone causes a dose-dependent reduction of FAK phosphorylation, a key protein in the focal adhesion complex, suggesting possible interference with cytoskeletal organization and cell adhesion modifications required to ensure complete execution of the mitogenic and chemotactic action of PDGF. The possible dependence of these effects on an alteration of Na^+/H^+ exchanger or antiporter activity is suggested by the fact that preincubation with EIPA causes similar inhibitory effects on PDGF-induced FAK phosphorylation.

Together, these data confirm that drugs comprising spironolactone compounds, particularly canrenone, potassium canrenoate, spironolactone and eplerenone, which are antialdosterone agents, can have an antifibrogenic effect by means of an action that occurs at the level of the intracellular signaling cascade induced by stimulation on the part of PDGF. An

antifibrogenic action of canrenone is also suggested by the inhibitory effect on de novo synthesis of extracellular matrix components, such as procollagen type I and fibronectin, induced by TGF-β.

Finally, in view of the results of the experiments in which canrenone proved itself capable of inhibiting the biological effects of PDGF in human smooth muscle cells, and since the pathophysiological mechanisms that are the basis of disorders characterized by progressive formation of fibrous tissue (including the atherosclerotic process) are quite similar, the results of this set of experiments provide an experimental basis for extending these findings to other cell types responsible for the onset of sclerosis in other organs and systems.

The disclosures in Italian Patent Application No. MI2001A000821 from which this application claims priority are incorporated herein by reference.

CLAIMS

1. Use of a spironolactone compound for manufacturing a medicament for inhibiting the pro-fibrogenic action of hepatic cells and vascular smooth muscle stellate cells.

2. The use according to claim 1, where said compound is selected from the group constituted by canrenone, potassium canrenoate, spironolactone itself and eplerenone.

3. The use according to claim 2, wherein said compound is canrenone.

4. The use according to claim 1, wherein said fibrogenesis is mediated by pro-fibrogenic cytokines.

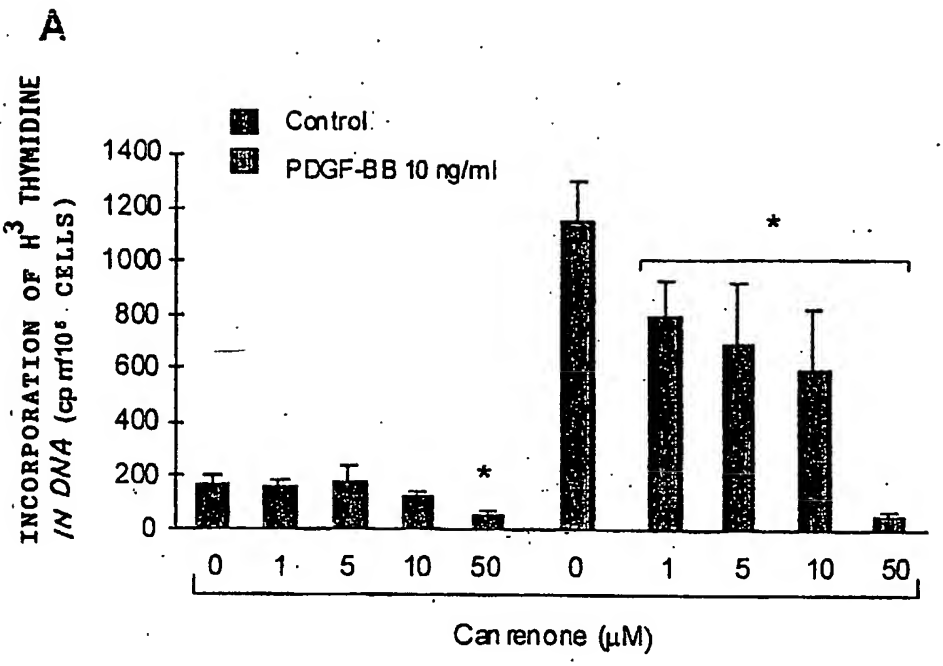
5. The use according to claim 4, wherein said fibrogenesis is mediated by PDGF and/or TGF-β.

6. The use according to claim 1, wherein said medicament is for administration in a quantity that is therapeutically effective in order to inhibit the fibrogenic process and is comprised within the dose range commonly used clinically for other indications.

7. The use according to claim 6, wherein said quantity is in the range from 50 to 100 mg/day.

8. The use of a spironolactone compound for the development of new pharmacological entities aimed at the medical treatment of disorders mediated by cytokines and characterized by chronic inflammation and progressive fibrogenesis.

Fig 1


A
INCORPORATION OF H3 THYMIDINE IN DNA (cpm/10⁵ CELLS)
Control
PDGF-BB 10 ng/ml
1400
1200
1000
800
600
400
200
0
*
*
0 1 5 10 50 0 1 5 10 50
Canrenone (µM)

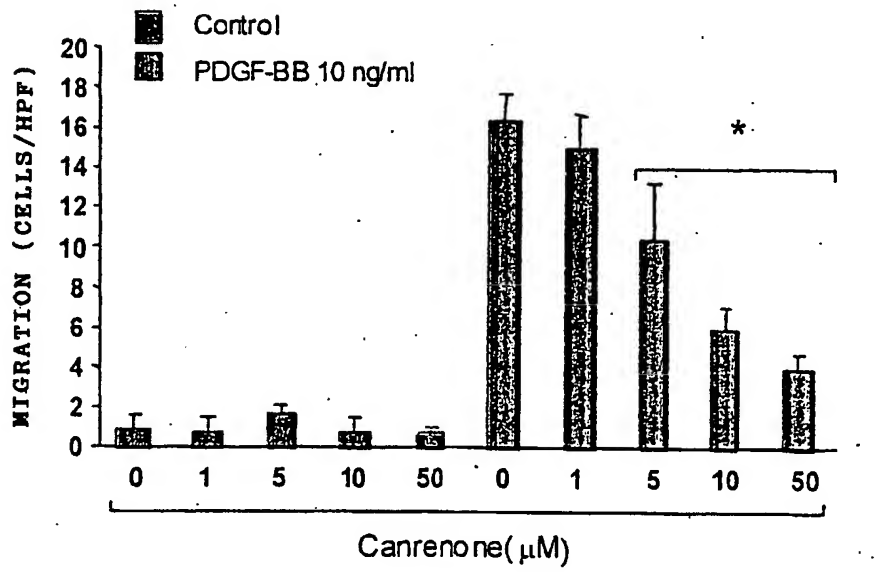
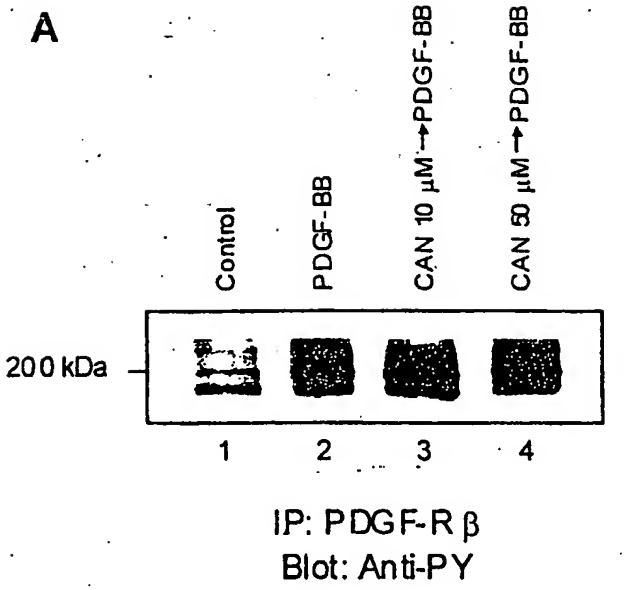

B
MIGRATION (CELLS/HPF)
Control
PDGF-BB 10 ng/ml
20
18
16
14
12
10
8
6
4
2
0
*
0 1 5 10 50 0 1 5 10 50
Canrenone(µM)

Fig 2


A
Control
PDGF-BB
CAN 10 µM→PDGF-BB
CAN 50 µM→PDGF-BB
200 kDa
1
2
3
4
IP: PDGF-R β
Blot: Anti-PY

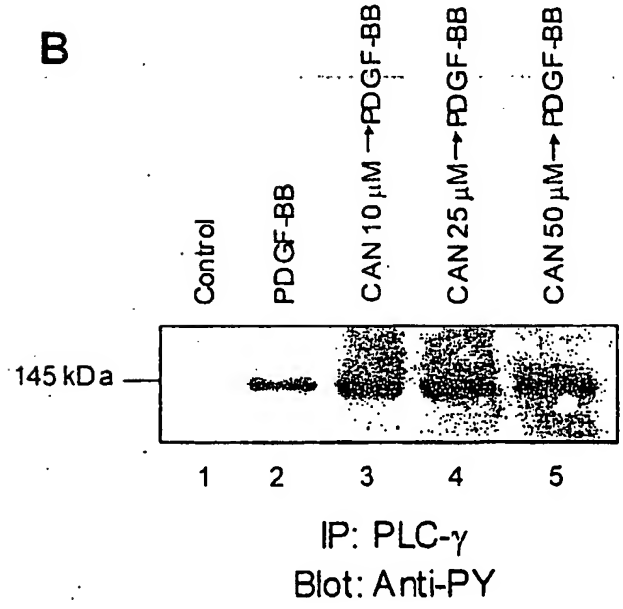
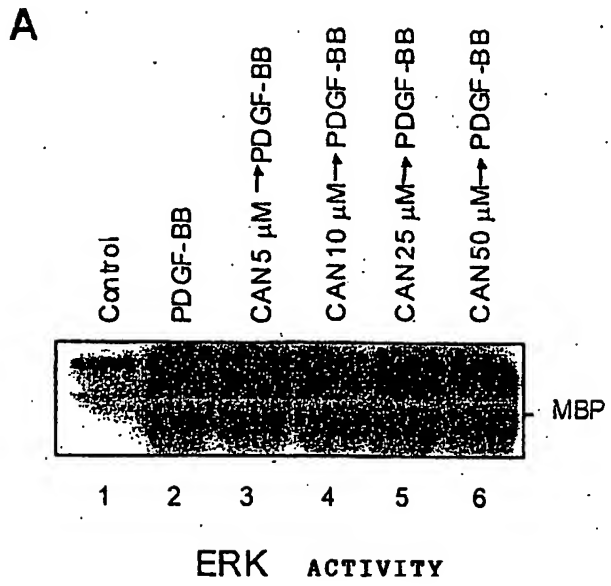

B
Control
PDGF-BB
CAN 10 µM→PDGF-BB
CAN 25 µM→PDGF-BB
CAN 50 µM→PDGF-BB
145 kDa
1
2
3
4
5
IP: PLC-γ
Blot: Anti-PY

Fig 3


A
Control
PDGF-BB
CAN5 µM →PDGF-BB
CAN10 µM→ PDGF-BB
CAN25 µM→ PDGF-BB
CAN50 µM→ PDGF-BB
MBP
1 2 3 4 5 6
ERK ACTIVITY

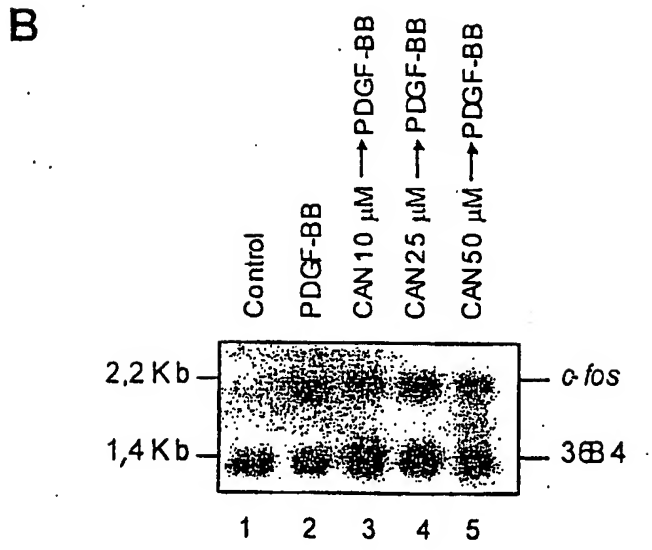

B
Control
PDGF-BB
CAN10 µM → PDGF-BB
CAN25 µM → PDGF-BB
CAN50 µM → PDGF-BB
2,2 Kb
1,4 Kb
c-fos
36B4
1 2 3 4 5

Fig 4

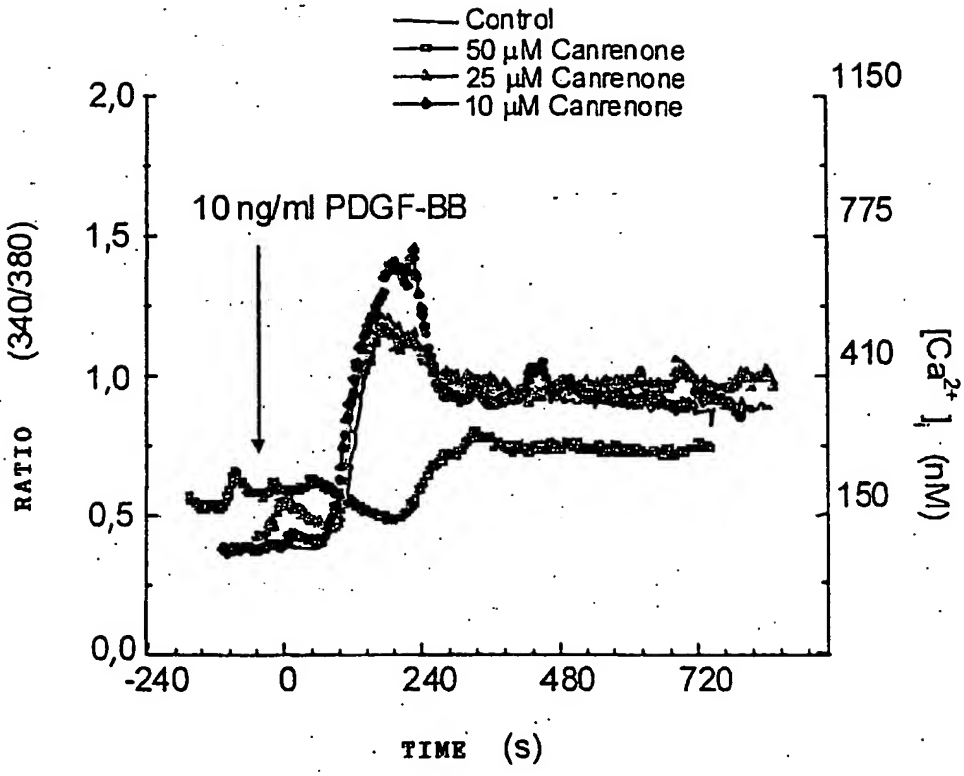

Control
50 µM Canrenone
25 µM Canrenone
10 µM Canrenone
10 ng/ml PDGF-BB
RATIO (340/380)
2,0
1,5
1,0
0,5
0,0
1150
775
410
150
[Ca2+]i (nM)
-240
0
240
480
720
TIME (S)

Fig 5

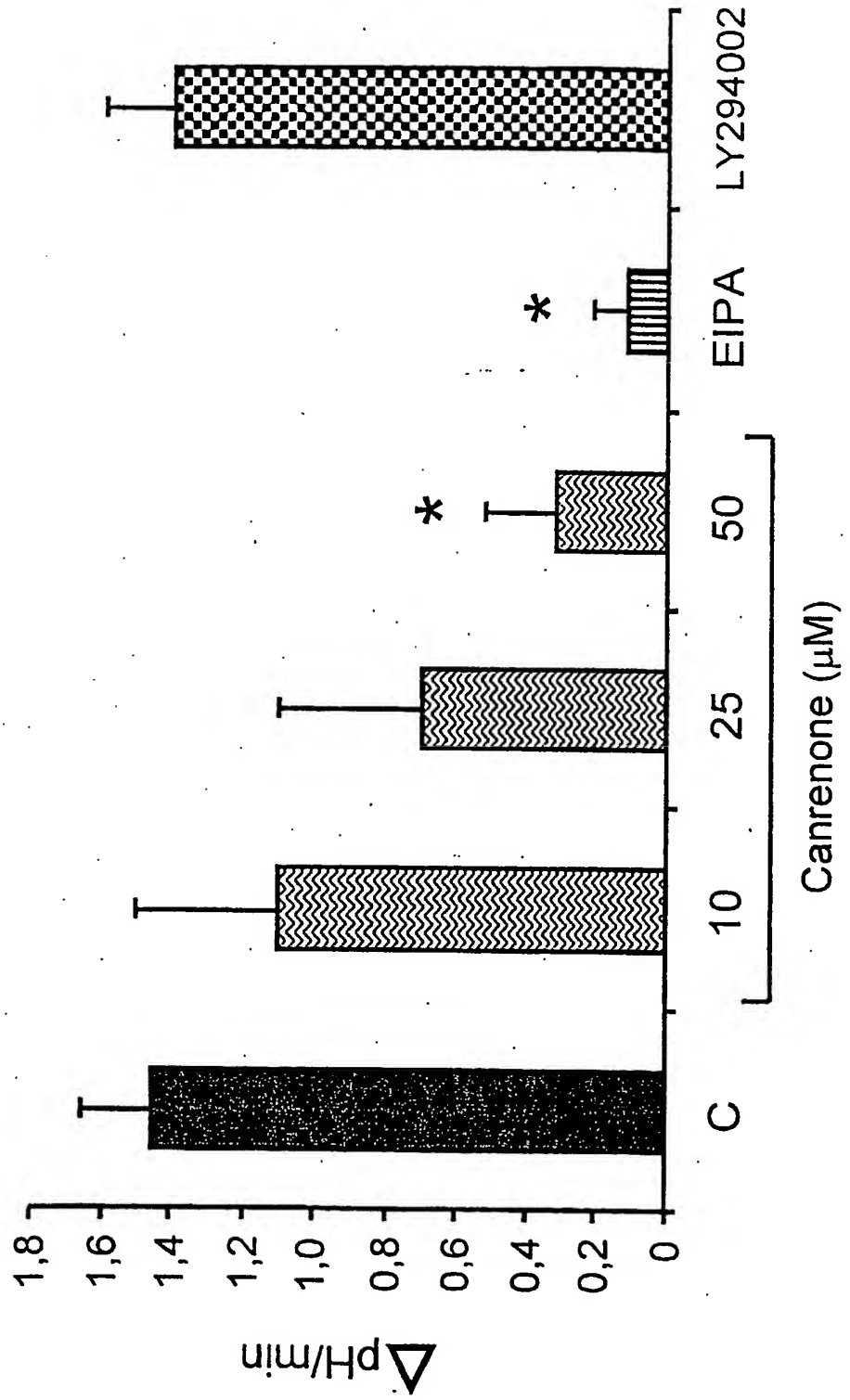

ΔpH/min
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0
C
10
25
50
Canrenone (μM)
*
*
EIPA
LY294002

Fig 6

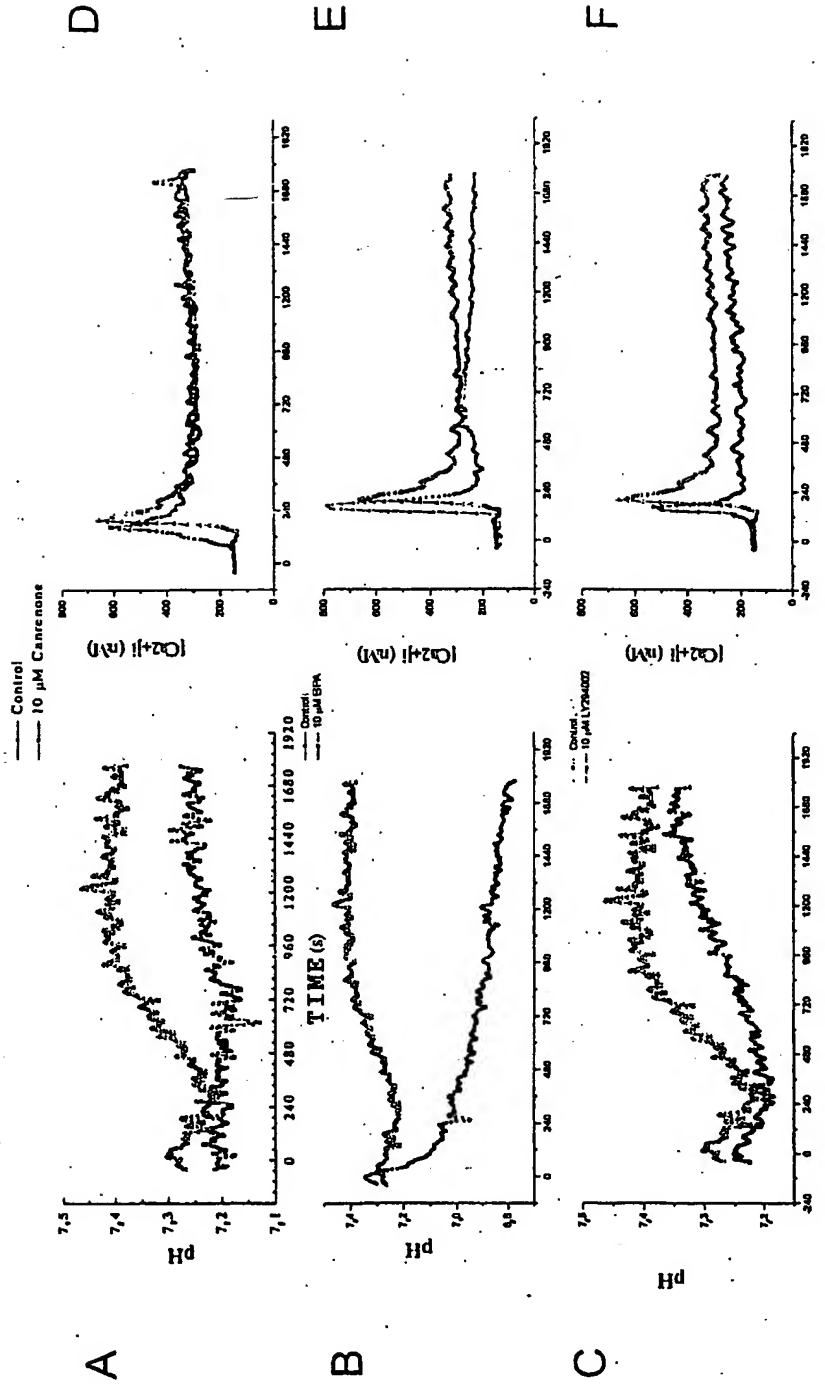
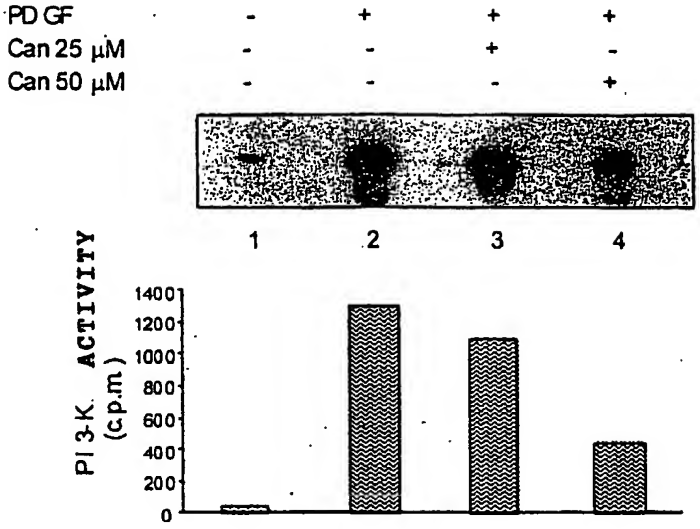

A
B
C
D
E
F
pH
[Ca2+]i (nM)
TIME(s)
Control
10 μM Carvenone
10 μM LY294002

Fig 7

A


PDGF - + + +
Can 25 µM - - + -
Can 50 µM - - - +
1 2 3 4
PI 3-K ACTIVITY (c.p.m.)
1400
1200
1000
800
600
400
200
0

B

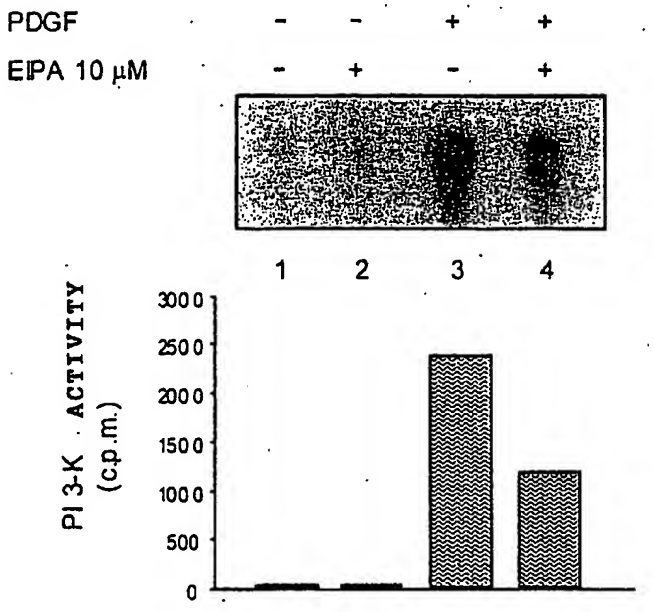
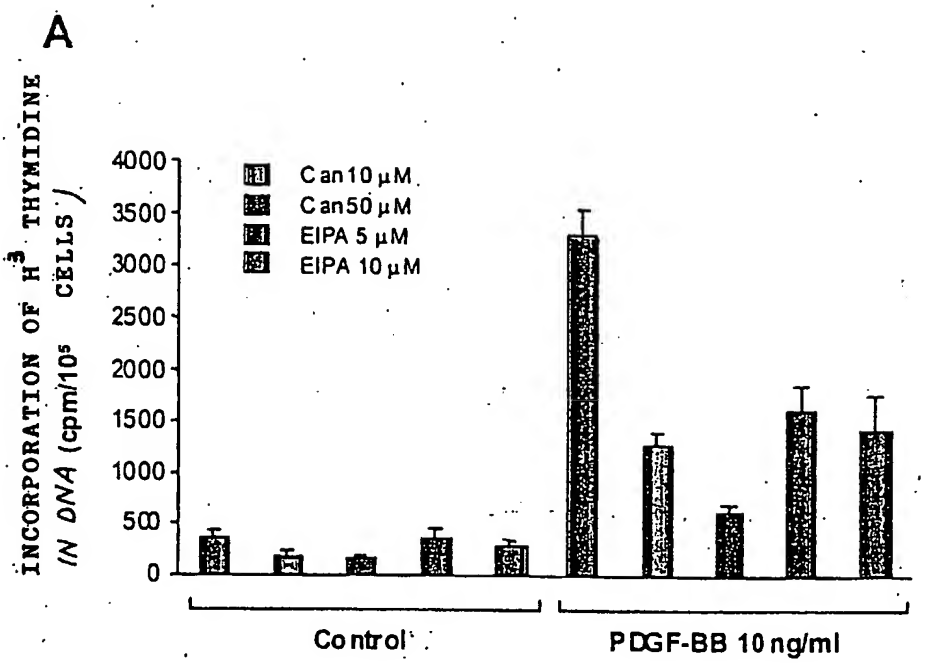
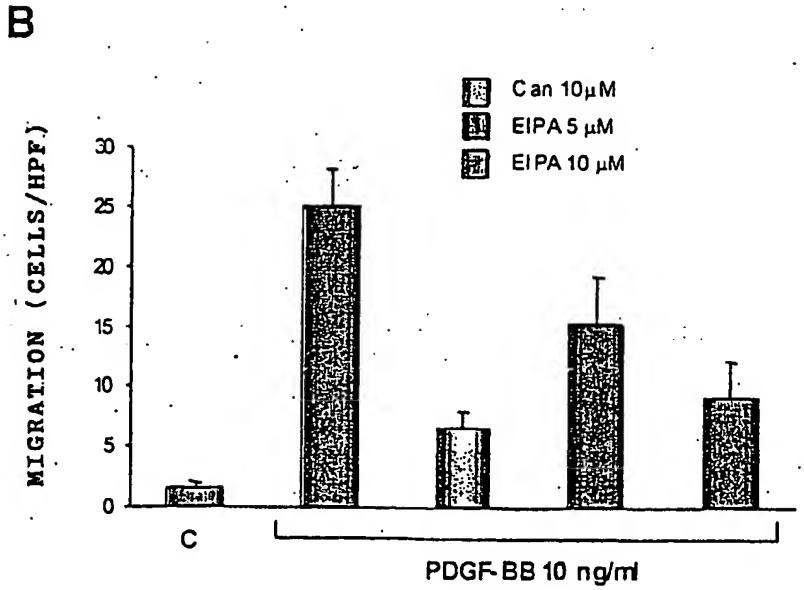

PDGF - - + +
EIPA 10 µM - + - +
1 2 3 4
PI 3-K ACTIVITY (c.p.m.)
3000
2500
2000
1500
1000
500
0

Fig 8


A
INCORPORATION OF H^3 THYMIDINE IN DNA (cpm/10^5 CELLS)
4000
3500
3000
2500
2000
1500
1000
500
0
Can10 µM
Can50 µM
EIPA 5 µM
EIPA 10 µM
Control
PDGF-BB 10 ng/ml


B
MIGRATION (CELLS/HPF)
30
25
20
15
10
5
0
Can 10µM
EIPA 5 µM
EIPA 10 µM
C
PDGF-BB 10 ng/ml

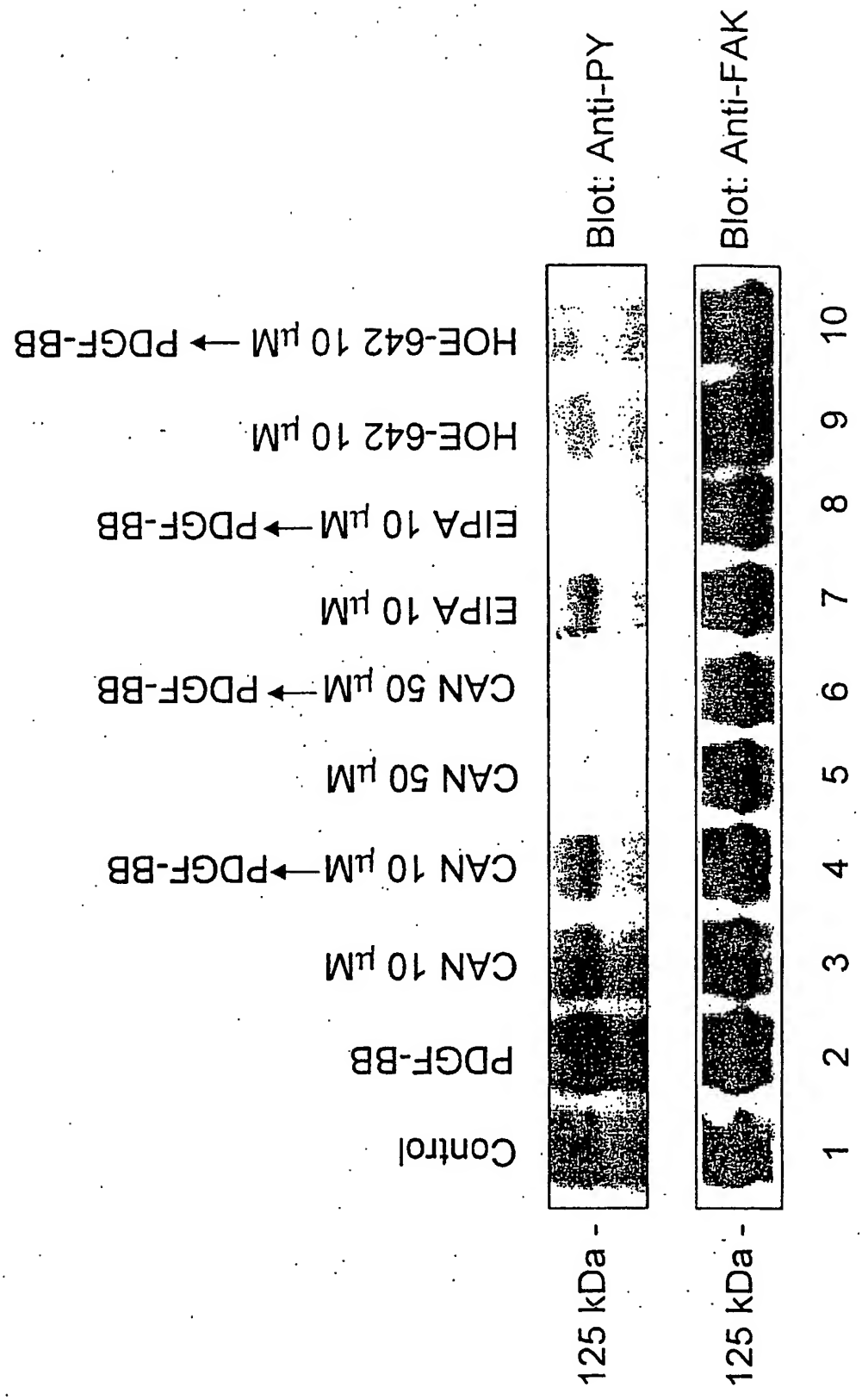

Blot: Anti-PY
Blot: Anti-FAK
125 kDa -
125 kDa -
1 2 3 4 5 6 7 8 9 10
IP: FAK
Control
PDGF-BB
CAN 10 µM
CAN 10 µM → PDGF-BB
CAN 50 µM
CAN 50 µM → PDGF-BB
EIPA 10 µM
EIPA 10 µM → PDGF-BB
HOE-642 10 µM
HOE-642 10 µM → PDGF-BB

Fig. 9

Fig 10

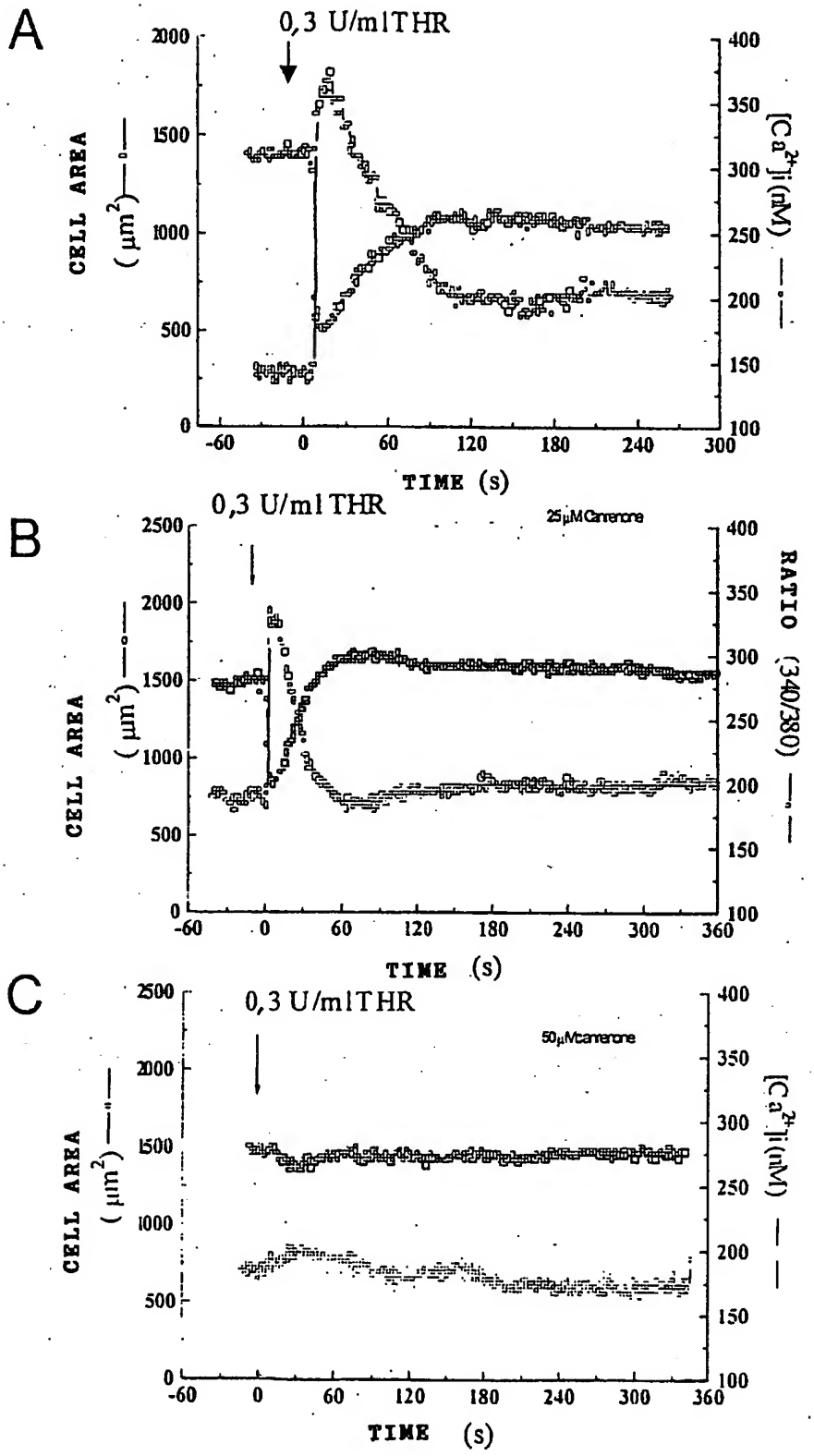

A
0,3 U/ml THR
CELL AREA (μm²)
[Ca²⁺]i (nM)
TIME (s)
B
0,3 U/ml THR
25μM Carnerone
CELL AREA (μm²)
RATIO (340/380)
TIME (s)
C
0,3 U/ml THR
50μM Carnerone
CELL AREA (μm²)
[Ca²⁺]i (nM)
TIME (s)

Fig 11A

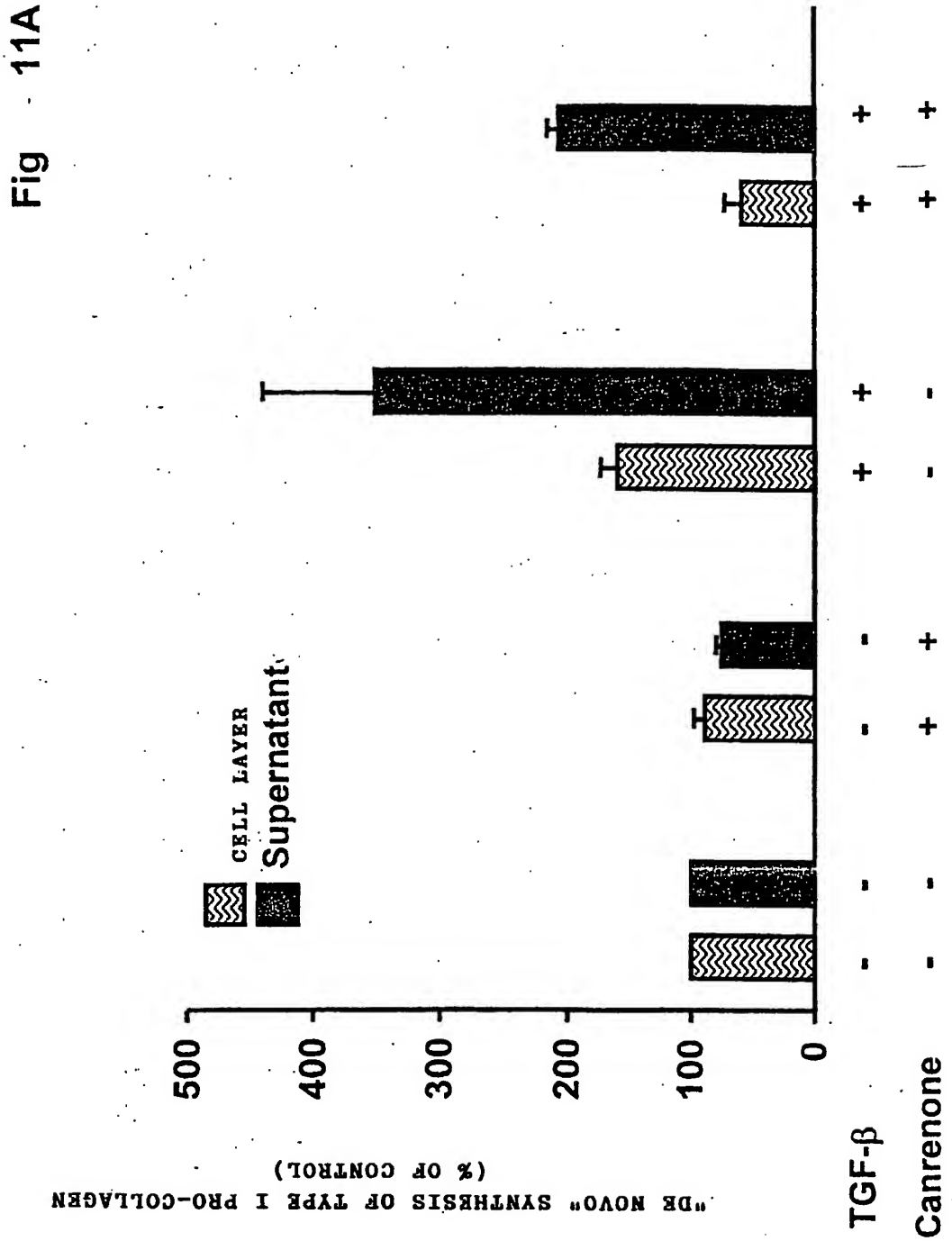

"DE NOVO" SYNTHESIS OF TYPE I PRO-COLLAGEN
(% OF CONTROL)
500
400
300
200
100
0
CELL LAYER
Supernatant
TGF-β
Canrenone
+ +
+ +
+ -
+ -
- +
- +
- -
- -

Fig 11B

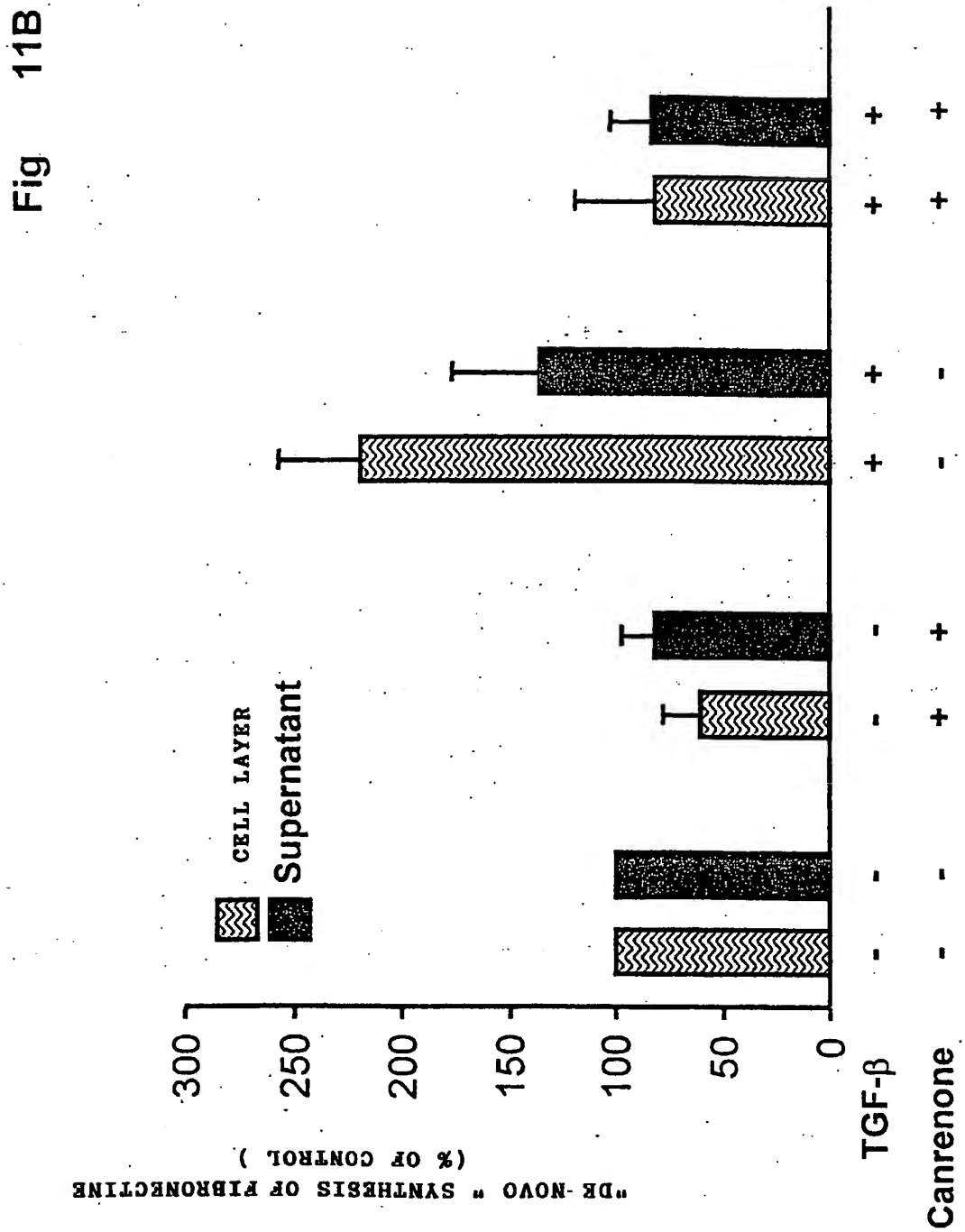

"DE-NOVO " SYNTHESIS OF FIBRONECTINE
(% OF CONTROL)
300
250
200
150
100
50
0
CELL LAYER
Supernatant
TGF-β
Canrenone
- - + +
- + - +
- + + -
- + + -

Fig 11C

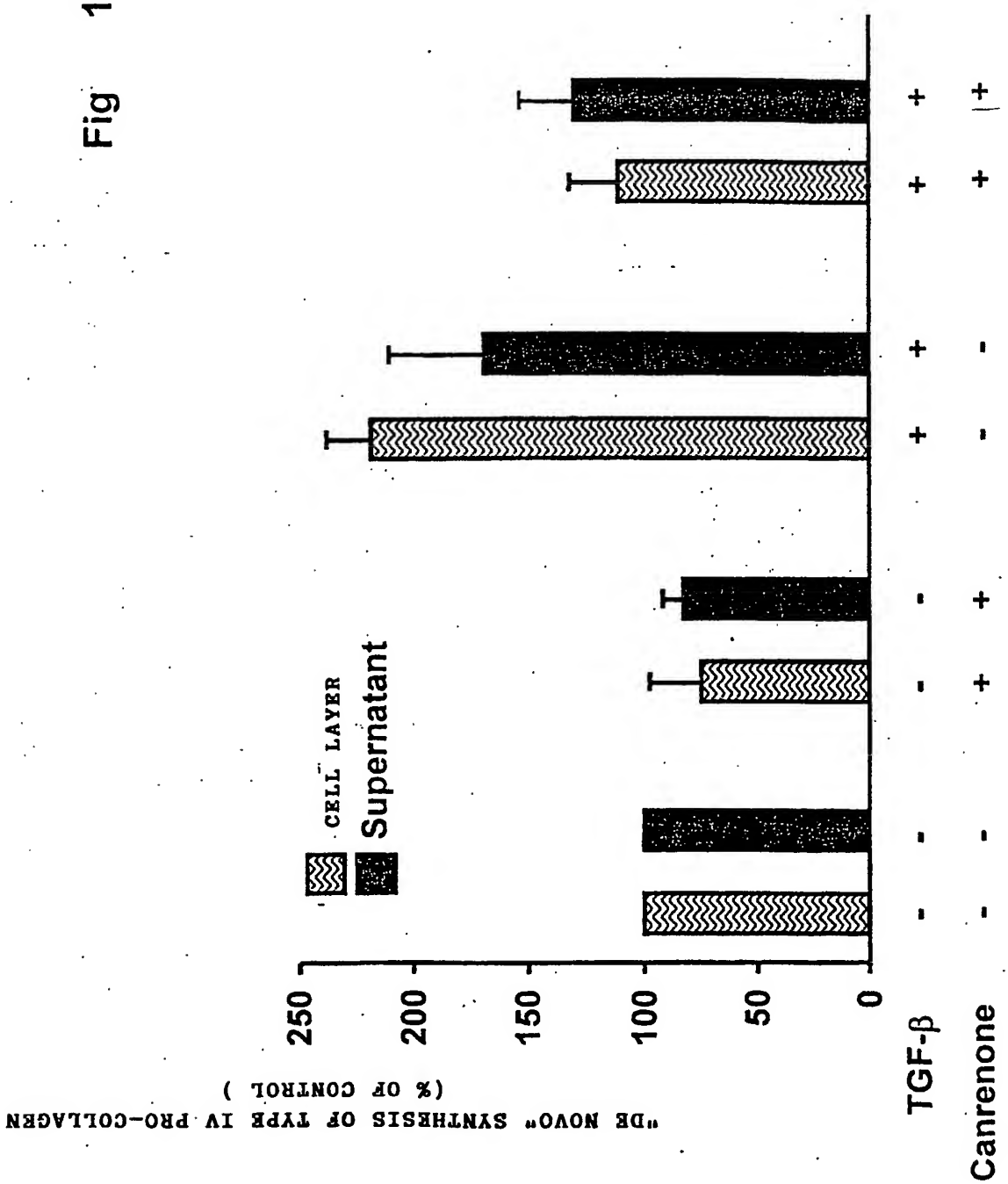

"DE NOVO" SYNTHESIS OF TYPE IV PRO-COLLAGEN
(% OF CONTROL)
250
200
150
100
50
0
CELL LAYER
Supernatant
TGF-β
Canrenone
+
-

Fig 12A

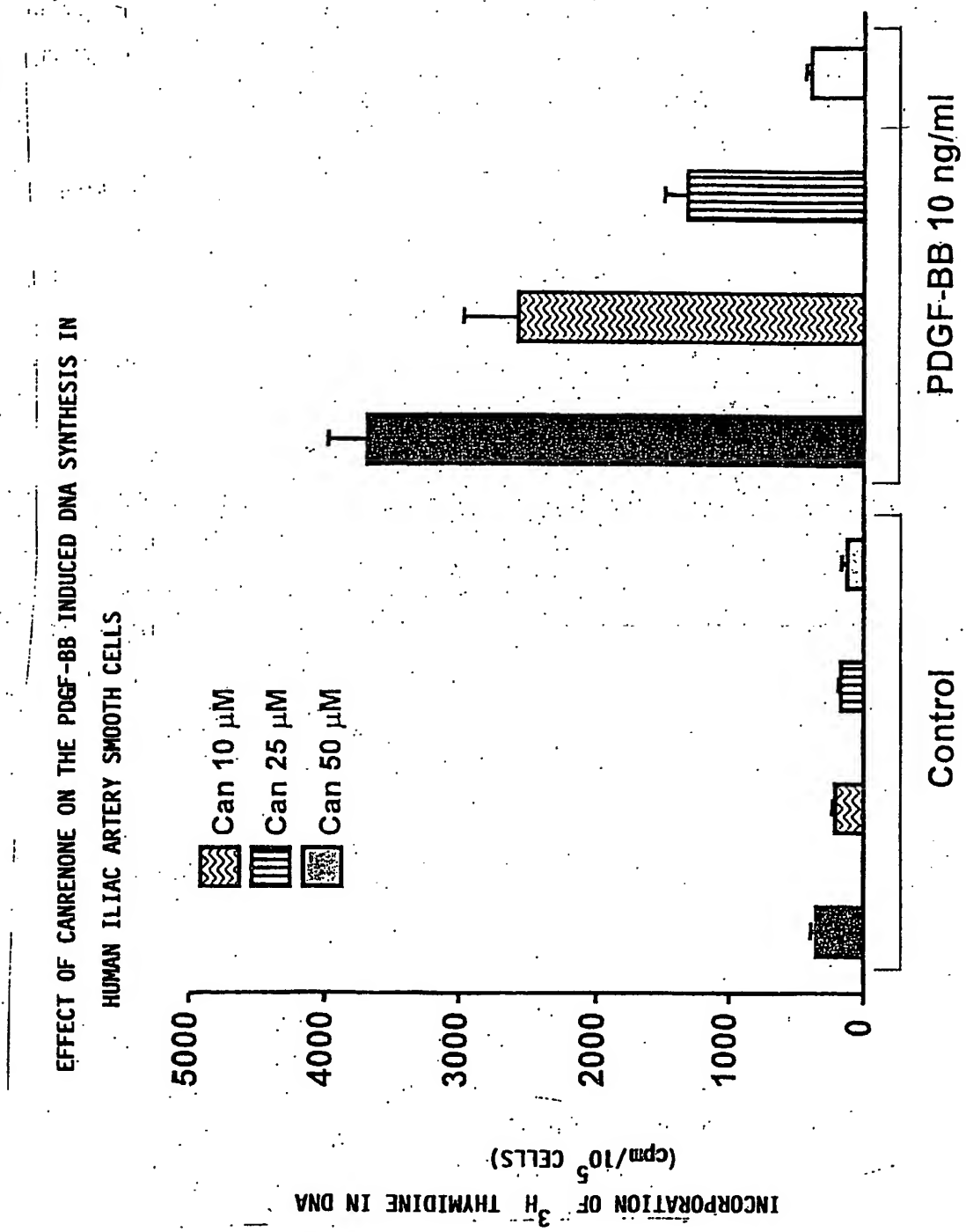

EFFECT OF CANRENONE ON THE PDGF-BB INDUCED DNA SYNTHESIS IN HUMAN ILIAC ARTERY SMOOTH CELLS
INCORPORATION OF ^{3}H THYMIDINE IN DNA (cpm/10^5 CELLS)
5000
4000
3000
2000
1000
0
Can 10 µM
Can 25 µM
Can 50 µM
Control
PDGF-BB 10 ng/ml

Fig 12B

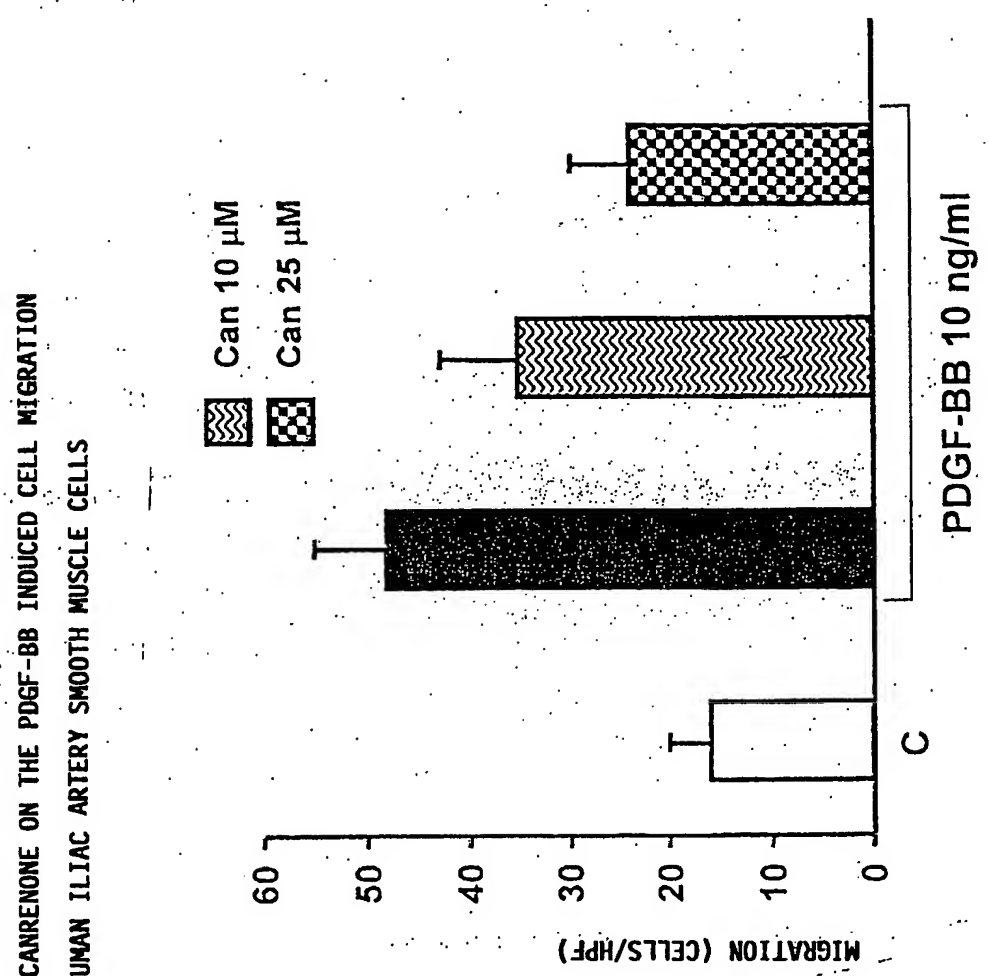
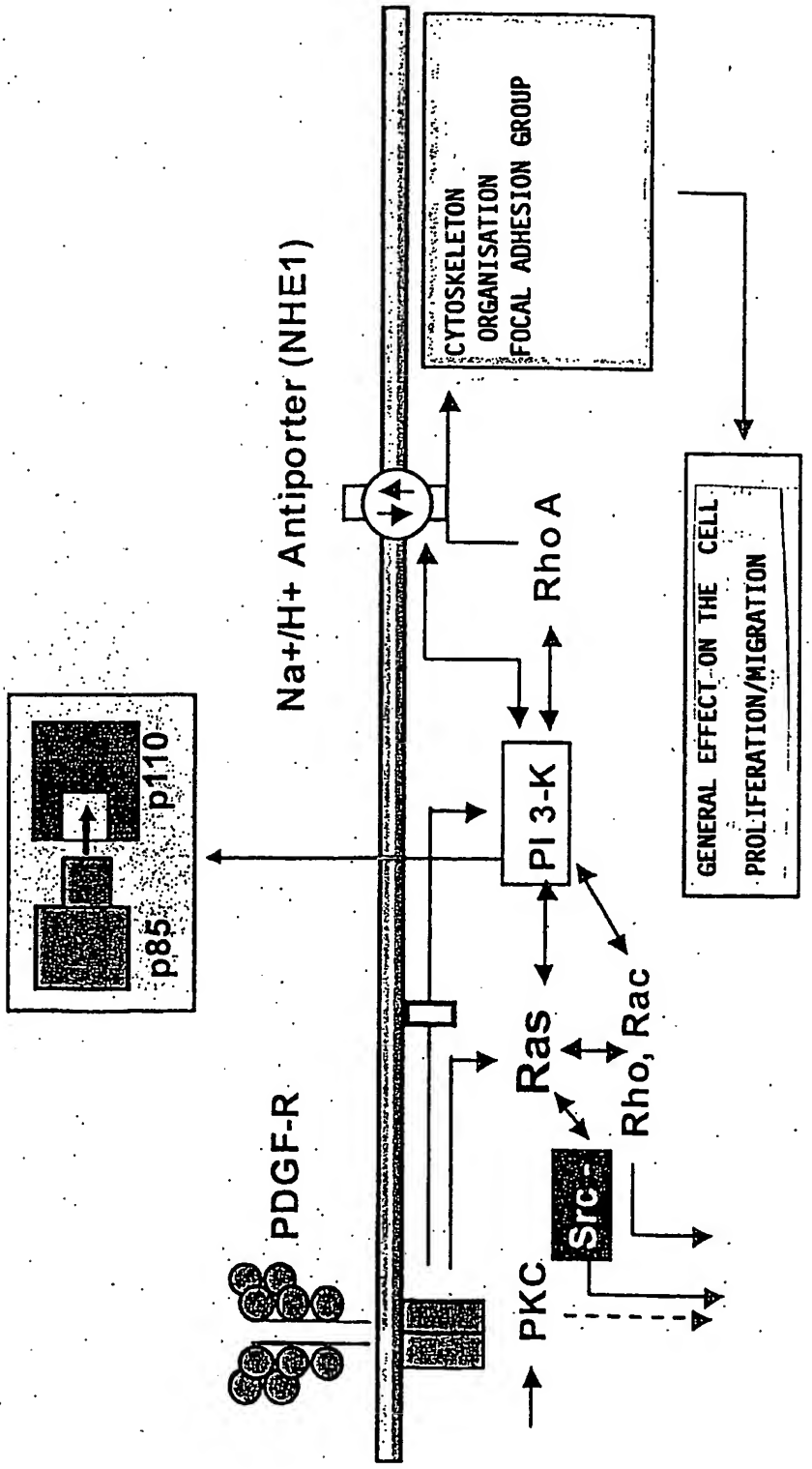

EFFECT OF CANRENONE ON THE PDGF-BB INDUCED CELL MIGRATION
IN HUMAN ILIAC ARTERY SMOOTH MUSCLE CELLS
Can 10 µM
Can 25 µM
MIGRATION (CELLS/HPF)
0
10
20
30
40
50
60
C
PDGF-BB 10 ng/ml

Fig 13


CYTOSKELETON
ORGANISATION
FOCAL ADHESION GROUP
GENERAL EFFECT ON THE CELL
PROLIFERATION/MIGRATION
Na+/H+ Antiporter (NHE1)
Rho A
PI 3-K
p110
p85
PDGF-R
Ras
Rho, Rac
Src
PKC